Taiwanese Journal of Obstetrics & Gynecology 56 (2017) 487–494

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Detection of SEA-type α-thalassemia in embryo biopsies by digital PCR

Ta-Hsien Lee [a], Ya-Chiung Hsu [b], Chia Lin Chang [b, *]

[a] Institute of Biomedical Science, Chang Gung University, No. 259, Wenhua 1st Road, Guishan Township, Taoyuan City 33302, Taiwan
[b] Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital Linkou Medical Center, Chang Gung University, 5 Fu-Shin Street, Gueishan Township, Taoyuan County 333, Taiwan

ARTICLE INFO

Article history:
Accepted 7 March 2017

Keywords:
α-thalassemia
SEA-Type
Embryo biopsy
Digital PCR
IVF

ABSTRACT

Objective: Accurate and efficient pre-implantation genetic diagnosis (PGD) based on the analysis of single or oligo-cells is needed for timely identification of embryos that are affected by deleterious genetic traits in *in vitro* fertilization (IVF) clinics. Polymerase chain reaction (PCR) is the backbone of modern genetic diagnoses, and a spectrum of PCR-based techniques have been used to detect various thalassemia mutations in prenatal diagnosis (PND) and PGD. Among thalassemias, SEA-type α-thalassemia is the most common variety found in Asia, and can lead to Bart's hydrops fetalis and serious maternal complications.

Materials and methods: To formulate an efficient digital PCR for clinical diagnosis of SEA-type α-thalassemia in cultured embryos, we conducted a pilot study to detect the α-globin and SEA-type deletion alleles in blastomere biopsies with a highly sensitive microfluidics-based digital PCR method. Genomic DNA from embryo biopsy samples were extracted, and crude DNA extracts were first amplified by a conventional PCR procedure followed by a nested PCR reaction with primers and probes that are designed for digital PCR amplification.

Results: Analysis of microfluidics-based PCR reactions showed that robust signals for normal α-globin and SEA-type deletion alleles, together with an internal control gene, can be routinely generated using crude embryo biopsies after a 10^6-fold dilution of primary PCR products.

Conclusion: The SEA-type deletion in cultured embryos can be sensitively diagnosed with the digital PCR procedure in clinics. The adoption of this robust PGD method could prevent the implantation of IVF embryos that are destined to develop Bart's hydrops fetalis in a timely manner. The results also help inform future development of a standard digital PCR procedure for cost-effective PGD of α-thalassemia in a standard IVF clinic.

Introduction

Alpha-thalassemia (α-thalassemia) is one of the most serious inherited autosomal diseases in Asian populations, and has a high prevalence in the tropical and sub-tropical areas. The majority of α-thalassemia can be attributed to genetic aberrations on chromosome 16p13.3 [1,2]. Large segment deletions of α-globin gene clusters cause defects in hemoglobin synthesis, and beget mild to severe anemia [3]. The most common α-thalassemias in South and Southeast Asia include the South-East Asian (SEA), Philippines (PHI), and Thailand (THAI) type deletions. Among them, the SEA-type deletion has the highest prevalence, and the carrier frequency ranges from 3 to 11% in most populations.

Most SEA-type carriers are asymptomatic or display mild symptoms, yet homozygous SEA-type deletion can lead to Hb Bart's hydrops fetalis in the offspring when both parents are carriers [4–7]. In addition, the presence of Hb Bart's hydrops fetalis can lead to serious maternal complications. Although the overall chance of having a baby with Hb Bart's hydrops fetalis is low in a population with a 3% prevalence of SEA-type carriers, it represents a serious concern for many couples, especially those with infertility issues. Because there is a one in four chance of having a baby with the Hb Bart's hydrops fetalis when both parents are carriers, it is prudent to diagnose potential homozygous embryos during the prenatal stage. This has led to a wide usage of prenatal diagnosis (PND) among high risk pregnant women, and later the application of pre-

* Corresponding author. Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital Linkou Medical Center, Chang Gung University, 5 Fu-Shin Street, Guishan Township, Taoyuan, Taiwan. Fax: +886-33288252.
E-mail address: amego@cgmh.org.tw (C.L. Chang).

http://dx.doi.org/10.1016/j.tjog.2017.03.002

implantation genetic diagnosis (PGD) in IVF patients prior to the beginning of pregnancy.

PGD, which examines the genetic makeup of pre-implantation embryos for inheritable gene defects, aims to prevent the possibility of transmitting genetic disorders among fertilized embryos in IVF clinics before embryo implantation, thereby allowing carriers of a genetically defective allele to conceive a healthy baby [8,9]. Most of the PND and PGD procedures utilize polymerase chain replication (PCR) to amplify the affected genetic component(s). These diagnoses use a wide spectrum of methods ranging from multiplex conventional PCR, real-time PCR, microsatellite PCR to droplet digital PCR [9–11]. Currently, the standard diagnosis for α-thalassemia carriers includes conventional PCR and real-time PCR. For example, studies using a multiple probe GAP-PCR approach indicated that this technique could allow 100% detection of SEA-type deletion in carriers and embryonic tissues [10]. Likewise, a recent study of multiplex fluorescent PCR demonstrated the possibility of using this technique to detect SEA-type deletion with genomic DNA from Hb Bart's hydrops fetalis and single-cell embryo samples [8,9]. However, the efficiency of obtaining acceptable amplification from single cells was only about 85%, and the method is still prone to erroneous readout, suggesting that a more reliable procedure that provides convenient and timely evaluation of select genotypes in cultured embryos is much needed.

Digital PCR is a high-resolution detection method that has been used in the detection of short-fragment tumor DNA in the blood of patients, and for non-invasive prenatal diagnosis of inheritable disorders [12–14]. Because the readout of digital PCR is based on signals in thousands of independent PCR reaction compartments, its readout is less prone to influence by contaminants when compared to single-tube PCR reactions [12,15]. It is also less affected by the amplification efficiency when compared to other PCR procedures [12,15]. Therefore, it could be an optimal tool for quick and accurate detection of inherited genetic disorders in cultured embryos at IVF clinics. While digital PCR is ideal for the quantification of individual targets in complex samples, the power of this method is limited in cases where known targets are relatively simple. To take advantage of the power of digital PCR, in the present study we evaluated the feasibility and optimal conditions of using a nested digital PCR approach to identify embryos with the SEA-type deletion allele before embryo transfer in an IVF clinic. The results may help inform future application of digital PCR for robust and cost-effective PGD of α-thalassemias in IVF clinics and reduce the occurrence of Hb Bart's hydrops fetalis [11].

Materials and methods

Patients, sample collection, and DNA extraction

All studies were conducted with the approval of the Institutional Review Board (IRB) and Human Research Ethics Committee at Chang Gung Memorial Hospital, and with informed consent from each patient. The IRB No. is 102-0050C. This study enrolled a total of 20 cases from March 2010 to May 2014. These patients aged 29–39, and have an average age of 33. Nineteen of these cases were normal, and only one couple carried the SEA-type thalassemia in both the maternal and paternal genomes. From this couple, we obtained 9 samples of 3 pro-nuclei (PN) zygotes, 27 samples of Day 3 arrested 2 PN embryos, and 4 samples of blastomere biopsies.

Biopsied blastomere(s) from the cleavage-stage or trophectoderm cell-stage embryos were collected into 0.2 ml Eppendorf tubes with 3 μl cell lysis buffer (Universal ^{TM} extraction buffer, Yeastern biotech CO. Ltd.) [16]. DNA was released following heating at 95 °C for 5 min, and all DNA samples were kept at −20 °C before the assay. The α-thalassemia positive control DNA samples were collected from the peripheral blood of well characterized SEA-type deletion carriers, and were extracted with the QIAamp DNA mini-kit (Qiagen, Germany). The concentration of DNA was quantified with a Thermo Nanodrop 1000 spectrophotometer.

Detection with the conventional PCR procedure

To identify normal α-globin and SEA-type deletion alleles, DNA from all blood and embryo biopsy samples were amplified with a nested PCR protocol (Fig. 1, Table 1). DNA samples were first amplified with a set of flanking PCR primers. The amplification was performed with the Qiagen HotstarTaq Plus DNA polymerase kit. The reactions each contained 2.5 μl 10X PCR buffer, 1 μl $MgCl_2$ (25 mM), 0.5 μl HotstarTaq Plus DNA polymerase (5 U/μl), 5 μl Q

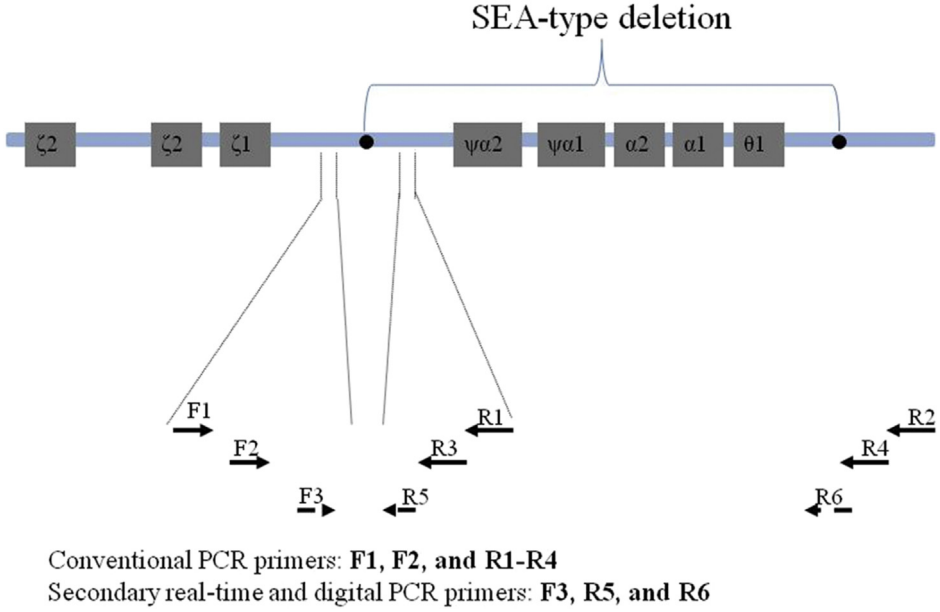

Fig. 1. Design of nested PCR primers and probes for the detection of SEA-type deletion in DNA of embryo biopsies. The positions of primary and secondary primers within the α-globin gene clusters are indicated by arrows. The position of SEA-type deletion is indicated by an arrow bracket.

Table 1
List of PCR primers and TaqMan probes.

	Primer Sequence (5' -> 3′)
Primers for primary PCR	
Forward F1	CTC TGT GTT CTC AGT ATT GGA GGG AAG GAG
Reverse R1 for normal α-globin	TGA AGA GCC TGC AGG ACC AGG TCA GTG ACC G
Reverse R2 for SEA-type deletion	ATA TAT GGG TCT GGA AGT GTA TCC CTC CCA
HS40 forward primer	TTC TGC AAC CAT GAT GAC TGG GTC AAA G
HS40 reverse primer	CTG AAG CCT GGC TGT GAA CAC TTT GG
Primers for secondary conventional PCR	
Forward F2	GCT TCG CAG GAA CTC GGT CGT CC
Reverse R3 for normal α-globin	TGC AGA CTT TAG GGG CAT CTG TTT ACT C
Reverse R4 for SEA-type deletion	GAG ACG ATG CTT GCT TTG TCA CCC ATG
HS40 forward primer	CAG TGC AGG AGG CTC TCA GGA ACA AGA
HS40 reverse primer	AGC ACC AGA GGT TGT AAT CAG CAG TAC CAT
Primers for real-time and digital PCR	
Forward F3	ACT CGG TCG TCC CCA CTG T
Reverse R5 for normal α-globin	CGC CCG TCC GAC TCA AG
Reverse R6 for SEA-type deletion	TGG CTT ACT GCA GCC TTG AA
TaqMan probes for real-time and digital PCR	**Reportor/Quencher Sequence (5' ->3′)**
Normal α-globin	**FAM-**CCC CTG AGC ACC GGA-**MGBNFQ**
SEA-type deletion	**FAM-**CAG GAG GAT CAC TTA AG-**MGBNFQ**
HS40	**Vic-**CCT ATC AGG GAC CAC AGT- **MGBNFQ**

solution, 1 μl dNTP (10 mM), 3 μl DNA sample and flanking primers (F1 + R1 or F1 + R2), and ddH_2O in a final volume of 25 μl. The amplification was carried out by heating to 95 °C for 5 min followed by 45 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 25 s. The first PCR products from blood DNA samples were diluted by 10^4-fold with ddH_2O, and amplified with a pair of allele-specific nested primers in the second PCR reaction (F2 + R3 or F2 + R4). For the second amplification of DNA from embryo biopsy samples, PCR products from the first amplification were routinely diluted 10-fold. The secondary PCR was conducted with the following protocol: heating to 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 25 s.

Quantification of the primary PCR products by a secondary real-time PCR

To evaluate DNA contents that are suitable for efficient detection of the normal α-globin and the SEA-type deletion alleles by digital PCR, we first quantify the amplifiable DNA in the first PCR reaction of embryonic DNA samples using a real-time PCR approach. Following the first PCR with the conventional method, the PCR products were diluted by 10^4- to 10^8-fold, and then amplified with a LightCycler® Real-Time PCR Capillary System (Roche, Germany) with nested PCR primers (Fig. 1). The reaction included 1x TaqMan master mix (Roche), nested PCR primers or a pair of internal control primers for the human α-globin upstream regulatory element sequence (HS-40), allele-specific probes (Table 1), and ddH_2O in a final volume of 20 μl. The amplification was conducted at 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s and 50 °C for 60 s.

Genotyping with the digital PCR procedure

To quantify the diagnosis of normal α-globin and the SEA-type deletion alleles in embryo biopsy samples, digital PCR was performed with a QuantStudio™ 3D Digital PCR System after primary

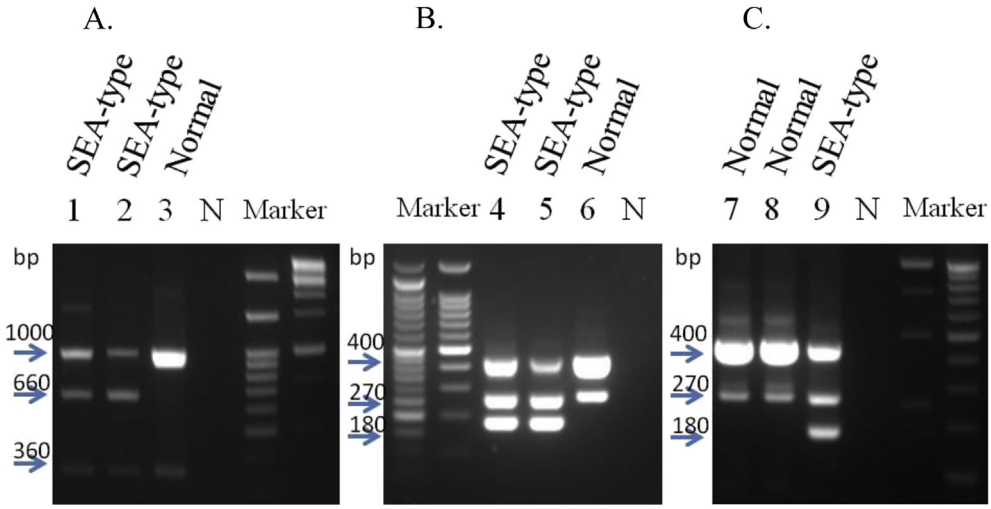

Fig. 2. Detection of normal α-globin and SEA-type deletion alleles by the conventional PCR approach. A. Genomic DNA from normal individuals and carriers of SEA-type α-thalassemia were initially amplified by a set of primary PCR primers (F1 + R1 + R2). DNA products from normal individuals (sample 3, a 1-kb band for normal α-globin and a 360-bp band for HS40) and SEA-type deletion carriers (samples 1 and 2, with one extra 660-bp band for the SEA-type deletion allele) displayed two and three faint bands, respectively. **B.** This result was confirmed by the secondary amplification with nested primers (F2 + R3 + R4). DNA products from normal individuals (sample 6) displayed two specific bands: a 400-bp normal α-globin band and a 270-bp HS40 band. DNA from SEA-carriers generated three specific bands (samples 4 and 5, one each for the normal α-globin allele, the SEA-type deletion allele [180 bp], and HS40). **C.** Following the same procedure, analysis of DNA from embryo biopsies showed that embryos with normal α-globin genotype (samples 7 and 8), or heterozygous SEA-type deletion (sample 9) can be identified only after the secondary PCR. No specific band was identified after primary PCR (data not shown). Marker: molecular size marker; N: negative control. Specific size markers are indicated by arrows.

reaction. Each reaction consisted of QuantStudio™ 3D Digital PCR master mix v2 (Life Technologies), secondary primers for the normal α-globin (Fig. 1, primers F3 + R5) or SEA-type deletion (primers F3 + R6) alleles, genotype-specific TaqMan probe, 3 μl DNA sample, and ddH_2O in a final volume of 15 μl. The primers and probes used in digital PCR were the same as those used in real-time PCR. Each digital PCR chip was loaded with 10 μl of the PCR reaction mixture, and amplified by a GeneAmp™ 9700 PCR system. The amplification was conducted at 96 °C for 10 min, followed by 39 cycles of 60 °C for 120 s, 98 °C for 30 s, and 60 °C for 120 s. The results were analyzed with a QuantStudio™ 3D Digital PCR Instrument (Life Technologies).

Results

Identification of normal α-globin and SEA-type deletion alleles in DNA from blood and embryo biopsy samples with a conventional PCR procedure.

To examine whether digital PCR is a viable approach for the PGD of SEA-type α-thalassemia of cultured embryos, we first tested the primers, probes, and template DNA samples with a conventional PCR protocol. To ensure an accurate assay using low-copy DNA samples, we adopted a nested PCR amplification protocol with allele-specific primers (Table 1; Fig. 1).

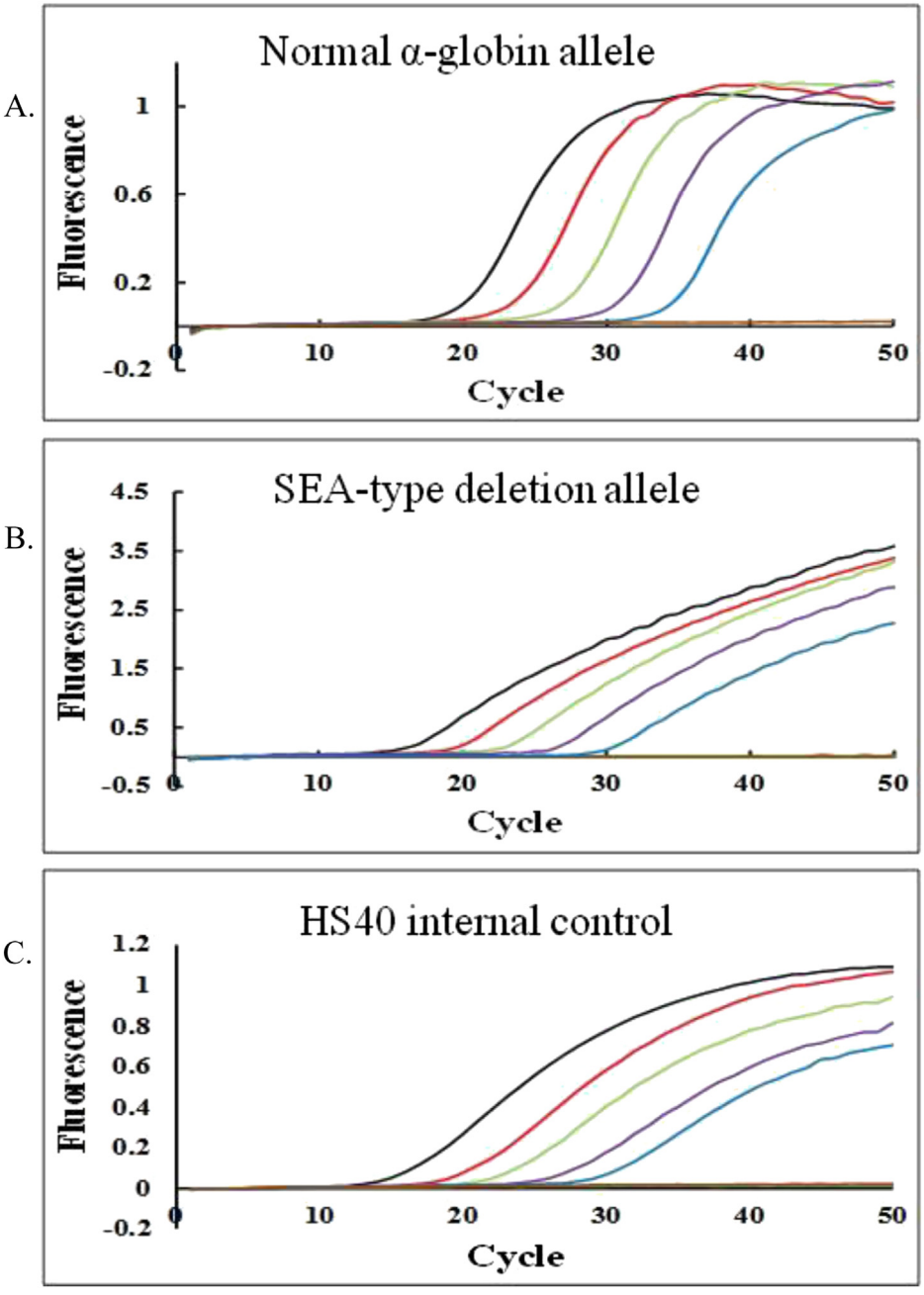

Fig. 3. Quantification of primary PCR products from embryo biopsy samples with real-time PCR. To calibrate the quantity of DNA that is suitable for the amplification by digital PCR, we amplified the primary PCR products of embryo biopsies using a set of digital PCR primers and TaqMan probes designed for the nested amplification of normal α-globin allele (**A**), SEA-type deletion allele (**B**), and HS40 internal control (**C**) with real-time PCR. The primary PCR products were diluted 10^4- to 10^8-fold, and the amplification curves showed that each of these genes were amplified in a dose-dependent manner.

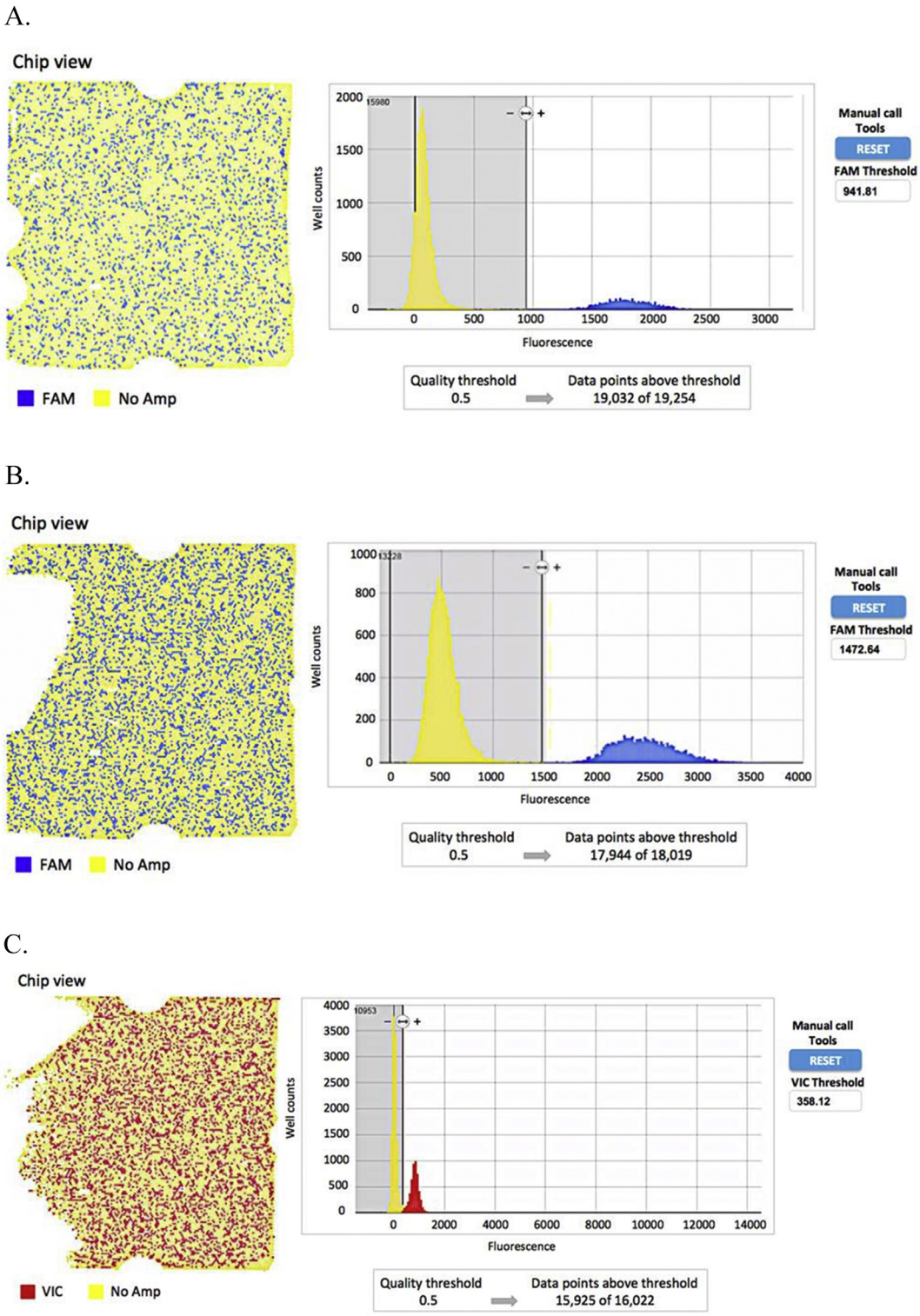

Fig. 4. Digital PCR amplification of normal α-globin allele and SEA-type deletion allele with DNA from embryo biopsies. The primary PCR products of embryo biopsies were amplified by digital PCR to generate normal α-globin (**A**, FAM labeling), SEA-type deletion (**B**, FAM labeling), HS40 (**C**, Vic labeling), normal α-globin and HS40 (**D**), or SEA-type deletion and HS40 (**E**) signals after a 10^6-fold dilution. Sample false-color images of microfluidic digital PCR chips showed that obvious fluorescent blue dot (FAM) and red dot (Vic) signals were generated in > 15% of the reaction compartments. In these images, a color dot represents a reaction compartment that gives off a signal. Discrete fluorescent signals were obtained when one (**A**–**C**) or two (**D**-**E**) sets of primers and probes were used in amplification. In reactions with two products (**D** and **E**), a green dot represents an overlap of blue dot (FAM) and red dot (Vic) signals in the same compartment.

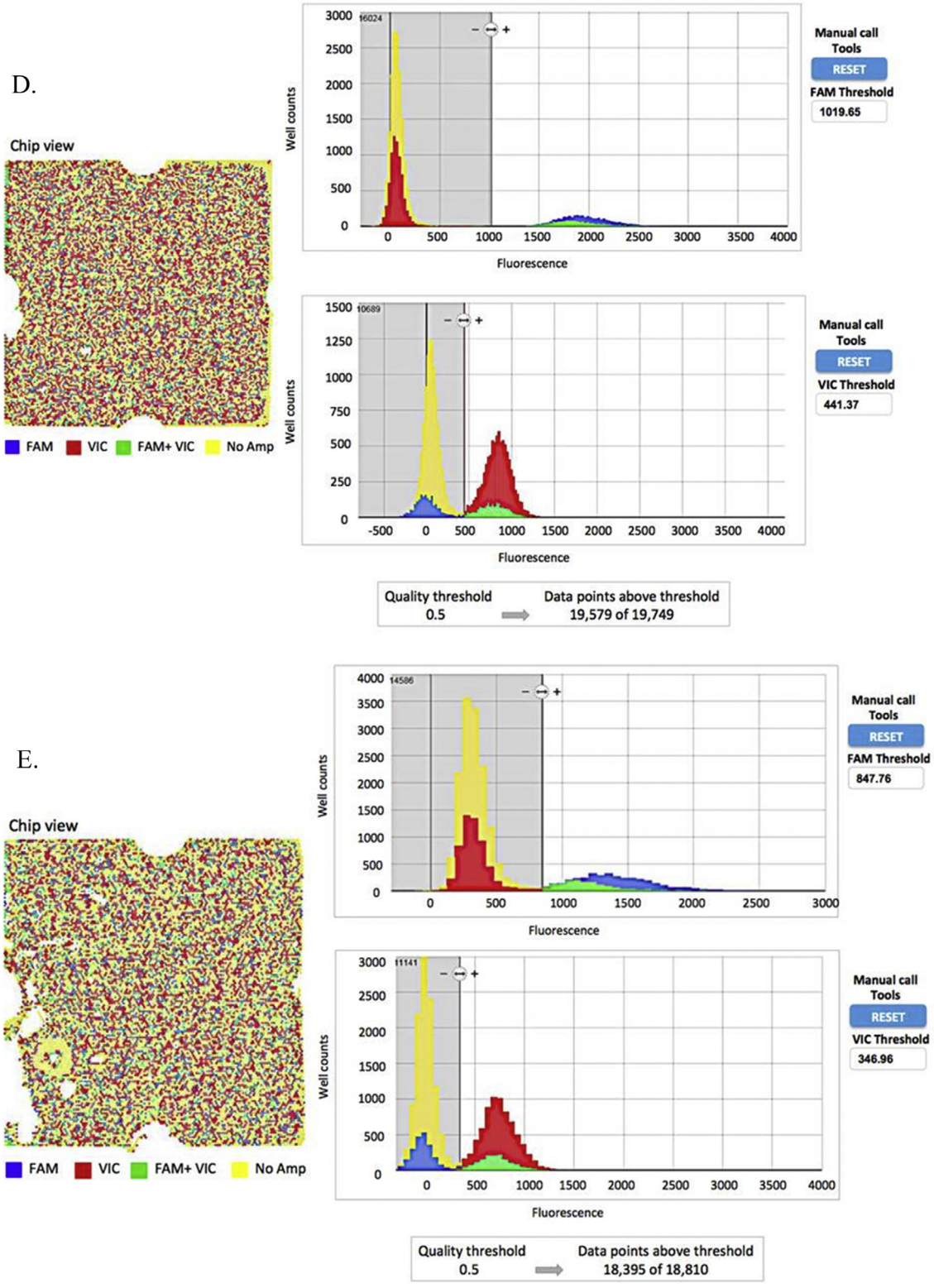

Fig. 4. (*continued*).

All DNA samples were first amplified with the flanking primers, followed by a second PCR reaction using nested primer pairs. As shown in Fig. 2, conventional PCR amplification of blood DNA samples from two carriers generated specific bands for both normal α-globin gene and SEA-type deletion in both primary PCR (Fig. 2A, samples 1 and 2) and secondary PCR (Fig. 2B, samples 4 and 5). On the other hand, DNA from a normal individual yielded only bands from normal α-globin gene in the primary and secondary PCR products (Fig. 2A, samples 3 and Fig. 2B, sample 6). Unlike blood genomic DNA samples, amplification of DNA from embryo biopsies

Table 2
Quantification of digital PCR signals from blastomere biopsy samples. An example of results from one embryo biopsy is presented.

Genotyping	Proportion and number of wells that are positive for select allele(s)	
	α-globin allele	HS40 control
One probe: normal α-globin allele	16% 3052/19032 wells	
One probe: SEA-type deletion allele	26.3% 4716/17944 wells	
One probe: HS40 control		31.2% 4972/15925 wells
Two probes: normal α-globin + HS40	18.2% 3555/19579 wells	45.4% 8890/19579 wells
Two probes: SEA-type deletion + HS40	20.1% 3809/18395 wells	39.4% 7254/18395 wells

generally yielded no specific bands or non-specific bands after the first PCR reactions (data not shown). Positive bands for the normal α-globin gene and/or the SEA-type deletion alleles were produced only after the secondary PCR (Fig. 2C; normal embryos, samples 7 and 8; carrier embryo, sample 9).

Quantification of pre-amplified DNA in embryo biopsy samples by real-time PCR

Because digital PCR is an extremely sensitive method for detecting low copy DNA, it is necessary to have an accurate assessment of the input DNA to allow optimal performance [12,15]. To do this, we evaluated the quantity of amplifiable DNA product in the first PCR reactions using a real-time PCR procedure. We amplified the three targeted PCR products (i.e., normal α-globin gene, SEA-type deletion, and the HS40 internal control) with five different dilutions of the first PCR products (10^4- to 10^8-fold dilution) using a Roche Light Cycler Capillary System. As shown in Fig. 3, the products of normal α-globin, SEA-type deletion, and the HS40 internal control were detected dose-dependently with regular Delta C_T curves. The results showed that the pre-amplified DNA products from blastomere DNA samples were relatively high, and a 10^5- to 10^6-fold dilution, or a C_T value between 27 and 30, could be suitable for the detection using a digital PCR approach. Accordingly, we selected a 10^6-fold dilution as the starting point for testing the digital PCR genotyping approach.

Preimplantation genetic diagnosis of SEA-type α-thalassemia in cultured embryos with digital PCR.

To evaluate the efficiency of digital PCR for the detection of SEA-type α-thalassemia in embryo biopsies, we analyzed the amplification of [1] normal α-globin allele [2], SEA-type deletion allele, and [3] HS40 internal control individually as well as [4] normal α-globin allele plus HS40 and [5] SEA-type deletion allele plus HS40 with templates that have been diluted 10^6-fold after the first PCR reaction.

Analysis of the digital PCR products with single gene probes showed that all reactions generated significant signals for the normal α-globin allele, SEA-type deletion allele, or the HS40 control sequences with high sensitivity (Fig. 4A–C). In these single probe reactions, 16–31.2% of amplification compartments (i.e., PCR reaction droplets) showed positive signals for the target genes (Table 2). Likewise, digital PCR with two different fluorescent probes detected the targeted α-globin gene allele and the HS40 control sequence simultaneously with high sensitivity (Fig. 4D and E). In these reactions, 18.2–45.4% of the amplification compartments showed positive signals (Table 2). Importantly, the signal intensity for α-globin gene alleles and that of the HS40 control in single and dual probe reactions are similar.

Discussions

Based on a nested PCR approach, we have shown that the SEA-type deletion in developing embryos can be efficiently diagnosed with digital PCR. In the protocol presented here, we were able to amplify normal and mutant α-globin alleles as efficient as a control gene with single- or oligo-copy of the genome in blastomere biopsies.

Homozygous α-globin gene defect can lead to Hb Bart's hydrops fetalis. Earlier studies have developed a variety of PCR approaches, including quantitative PCR, multiplex fluorescent PCR, multiplex-microsatellite PCR, real-time GAP-PCR, and droplet digital PCR to identify carriers of the SEA-type deletion allele [9–11, 17, 18]. Some of these methods have also been adopted for the diagnosis of SEA-type deletion in PGD, thus avoiding the consequences of implanting defective embryos.

Currently, conventional PCR is the accepted tool for determining the presence of SEA and other types of α-thalassemia in carriers or embryos in IVF clinic. Almost all current clinically applicable procedures were based on single-tube formats in a binary process regardless which PCR method was used. These traditional diagnostic strategies are prone to amplification failure, and such failure can lead to misdiagnosis. For example, a recent report using multiplex fluorescent PCR on heterozygote single cells indicated that the amplification efficiency with blastomere biopsy samples is approximately 85%, and erroneous results was obtained in select samples. Therefore, a more reliable test is desired.

Recent developments have suggested that digital PCR could be a more accurate method for the quantification of genetic disorders in complex samples with extremely low copy DNA. Microfluidics-based digital PCR is a method that counts amplification from single nucleic acid molecules in thousands of separate compartments [19, 20], and it converts the exponential nature of PCR to linear signals that can easily be measured with high precision. In addition, unlike conventional or real-time PCR, quantification with digital PCR is not affected by the efficiency of amplification and does not require a calibration curve [21]. A recent study has reported that droplet digital PCR is capable of detecting SEA-type thalassemia in carriers with high efficiency [11]. However, whether this approach is useful for PGD has not been described. Because blastomere biopsies contain only single or oligo copies of genome, we adopted a nested PCR approach to expand the templates in primary reactions, thereby allowing the presence of target alleles to be digitally quantified with a high confidence.

The utility of a PGD assay depends on a number of factors. An optimal assay requires high sensitivity and specificity, and can be performed in a timely manner. Because digital PCR enables rapid detection of the α-globin alleles from early embryos within a couple of hours after sampling and is not allele dependent, these data highlight the potential of using digital PCR to facilitate genetic diagnoses of SEA-type deletion and other genetic disorders in the cleaved-stage embryos (i.e., day 3 post-fertilization). Future studies that are aimed to simplify the digital PCR protocol for a speedy identification of affected embryos will be needed in order to make this method an accepted PGD assay for the SEA-type deletion.

In conclusion, the digital PCR appears to be a highly accurate method for the detection of SEA-type deletion in biopsied blastomere samples when it is performed with a nested PCR design. Because this technique requires minimal labor and has a rapid turnaround time, it could provide unforeseen benefits for routine diagnosis of α-thalassemia in IVF clinics.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding/Support Statement

This study was funded by Grants CMRPG3A0491-CMRPG3A0493 from Chang Gung Memorial Hospital.

References

[1] Higgs DR, Vickers MA, Wilkie AO, Pretorius IM, Jarman AP, Weatherall DJ. A review of the molecular genetics of the human alpha-globin gene cluster. Blood 1989 Apr;73(5):1081–104.
[2] Buckle VJ, Higgs DR, Wilkie AO, Super M, Weatherall DJ. Localisation of human alpha globin to 16p13.3––pter. J Med Genet 1988 Dec;25(12):847–9.
[3] Fucharoen G, Fucharoen S, Wanhakit C, Srithong W. Molecular basis of alpha (0)-thalassemia in northeast of Thailand. Southeast Asian J Trop Med public health 1995;26(Suppl. 1):249–51.
[4] Chen TP, Liu TC, Chang CS, Chang JG, Tsai HJ, Lin SF. PCR-based analysis of alpha-thalassemia in Southern Taiwan. Int J Hematol 2002 Apr;75(3):277–80.
[5] Chui DH, Waye JS. Hydrops fetalis caused by alpha-thalassemia: an emerging health care problem. Blood 1998 Apr 1;91(7):2213–22.
[6] Hsieh FJ, Ko TM, Chen HY. Hydrops fetalis caused by severe alpha-thalassemia. Early Hum Dev 1992 Jun-Jul;29(1–3):233–6.
[7] Ko TM, Hsieh FJ, Hsu PM, Lee TY. Molecular characterization of severe alpha-thalassemias causing hydrops fetalis in Taiwan. Am J Med Genet 1991 Jun 1;39(3):317–20.
[8] Piyamongkol W, Vutyavanich T, Sanguansermsri T. Preimplantation genetic diagnosis of alpha-thalassemia-SEA using novel multiplex fluorescent PCR. J Assist Reprod Genet 2012 Jan;29(1):95–102.
[9] Wang W, Yap CH, Loh SF, Tan ASC, Lim MN, Prasath EB, et al. Simplified PGD of common determinants of haemoglobin bart's hydrops fetalis syndrome using multiplex-microsatellite PCR. Reprod Biomed online 2010 Nov;21(5):642–8.
[10] Pornprasert S, Phusua A, Suanta S, Saetung R, Sanguansermsri T. Detection of alpha-thalassemia-1 Southeast Asian type using real-time gap-PCR with SYBR green1 and high resolution melting analysis. Eur J Haematol 2008 Jun;80(6):510–4.
[11] Pornprasert S, Prasing W. Detection of alpha(0)-thalassemia South-East Asian-type deletion by droplet digital PCR. Eur J Haematol 2014 Mar;92(3):244–8.
[12] Lo YM, Lun FM, Chan KC, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A 2007 Aug 7;104(32):13116–21.
[13] Karakas B, Qubbaj W, Al-Hassan S, Coskun S. Noninvasive digital detection of fetal DNA in plasma of 4-week-pregnant women following in vitro fertilization and embryo transfer. PLoS One 2015;10(5). e0126501.
[14] Jin S, Lin XM, Law H, Kwek KY, Yeo GS, Ding C. Further improvement in quantifying male fetal DNA in maternal plasma. Clin Chem 2012 Feb;58(2):465–8.
[15] Shen F, Du W, Kreutz JE, Fok A, Ismagilov RF. Digital PCR on a slipchip. Lab Chip 2010 Oct 21;10(20):2666–72.
[16] El-Hashemite N, Delhanty JD. A technique for eliminating allele specific amplification failure during DNA amplification of heterozygous cells for pre-implantation diagnosis. Mol Hum Reprod 1997 Nov;3(11):975–8.
[17] Chan V, Yip B, Lam YH, Tse HY, Wong HS, Chan TK. Quantitative polymerase chain reaction for the rapid prenatal diagnosis of homozygous alpha-thalassaemia (Hb Barts hydrops fetalis). Br J Haematol 2001 Nov;115(2):341–6.
[18] Sangkitporn SK, Wangkahat K, Sangnoi A, Songkharm B, Charoenporn P, Sangkitporn S. Rapid diagnosis of alpha(o)-thalassemia using the relative quantitative PCR and the dissociation curve analysis. Clin Lab Haematol 2003 Dec;25(6):359–65.
[19] Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999 Aug 3;96(16):9236–41.
[20] Kalinina O, Lebedeva I, Brown J, Silver J. Nanoliter scale PCR with TaqMan detection. Nucleic Acids Res 1997 May 15;25(10):1999–2004.
[21] Fan HC, Blumenfeld YJ, El-Sayed YY, Chueh J, Quake SR. Microfluidic digital PCR enables rapid prenatal diagnosis of fetal aneuploidy. Am J Obstet Gynecol 2009 May;200(5):543 e1–7.